In vitro glycation of glomerular basement membrane alters its permeability: a possible mechanism in diabetic complications

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Abstract The permeability of glomerular basement membrane (GBM) was assessed in vitro by the filtration of solutions of proteins across films formed from isolated pig GBM. Incubation of the films with fructose or glucose increased their permeability to water and serum albumin. The effect of fructose was similar to that previously noted for films crosslinked with glutaraldehyde. The metal chelator DTPA abolished the effects of glycation; EDTA was partially effective in this respect. Transition metal catalysed formation of glycoxidation induced crosslinks may explain the increased permeability of glycated GBM.

Key words: Glycation; Fructation; Diabetes; Diabetic nephropathy; Glomerular basement membrane

1. Introduction

There is strong evidence supporting the link between poor control of blood glucose and the development of diabetic complications [1]; diabetic nephropathy is one such major complication. The only continuous barrier of the glomerular capillary wall is the glomerular basement membrane (GBM) and changes in its structure may alter renal function. Does exposure of GBM to reducing sugars alter its molecular sieving properties? This question has been addressed by Daniels and Hauser [2] who found that exposure of GBM to glucose, in the presence of EDTA, did not alter its permeability. We have reinvestigated the effect of reducing sugars on GBM and find that both fructose and glucose alter GBM permselectivity but that glucose has less marked effects than fructose.

2. Materials and methods

2.1. Preparation of pig glomerular basement membrane

Pig kidneys were obtained from the abattoir and immediately chilled; thereafter procedures were at 0°C unless otherwise indicated. The cortices from 6–8 kidneys (approximately 500 g wet weight) were minced with a 'screw-type' mincer and using the base of a beaker were smeared through a 250 μm sieve in batches of approximately 50 g while washing with TBS (0.145 M NaCl, 0.01 M Tris-HCl buffer, pH 7.4, 0.02% (w/v) sodium azide). The residue of connective tissue was discarded and the combined washings were cycled three times through a 250 μm sieve until no lumps remained. The glomeruli were collected on a 63 μm sieve which was repeatedly partially immersed in buffer to flush the tubules through the sieve to yield glomeruli approximately 97% pure (as checked by light microscopy). The glomeruli were suspended in TBS containing 0.5% lauroyl sarcosine and homogenised using a top-drive homogeniser (Silverson Machines Ltd., Chesham, UK). The crude basement membrane was collected by centrifugation (1000 × g, 30 min) and the residue washed with detergent two more times. After the final detergent wash the pellet was washed in TBS buffer before being resuspended in approximately 300 ml of buffer and sonicated at maximum power in bursts of 1 min with cooling between each burst; sonication was continued until there were no recognisable glomeruli in the sample (usually after about 10 min of sonication time). The GBM was recovered by centrifugation and resuspended in 0.145 M NaCl with 5 mM $MgCl_2$ and 0.001% (w/v) DNase (No. 260912 Calbiochem, Nottingham, UK) and incubated at 20°C for 1 h. The GBM was washed in TBS 6 times and stored at 0°C. The amount of protein recovered was measured by dry weight and the yield averaged 50 mg.

2.2. Filtration studies

Filtration studies were performed as described previously [3]. A suspension of GBM fragments (1.5 mg dry weight) was filtered onto a Millipore membrane (HAWP 45 μm exclusion membranes, Millipore Ltd., London, UK) in an Amicon type 52 filtration cell (Amicon Ltd., Gloucestershire, UK) at pressures of 200 kPa until all the liquid had passed through the filter. The films so formed, composed of approximately 20 layers of interleaved basement membrane fragments, were then washed in TBS for 20 min at a pressure of 150 kPa. Films were mechanically stable, free from leaks and behaved reproducibly. Filtration cells were run in triplicate. To study the filtration of BSA, 50 ml of the protein solution (1 mg/ml) was put into each filtration cell and pressure (N_2) applied. Filtration of the solution was continued until approximately 8 ml of filtrate had been collected to allow the dead space in the filtration apparatus to reach equilibrium. Once equilibrium had been reached, timed collections of filtrate were begun, with volumes of between 1.0 and 1.5 ml being collected. Both the volume and the protein concentration (A_{280}) of each timed fraction were measured. In addition the volume of the initial filtrate and the protein concentrations of both the initial filtrate and the overstanding solution were measured. From these measurements it is possible to calculate the solvent flux (J_v), the solute flux (J_s) and the solute rejection ($\varepsilon = 1-(C_f/C_b)$), where C_f is the concentration of the filtrate and C_b is the concentration of the remaining solution corrected for changes during filtration.

Solutions of BSA were filtered at a range of gas pressures to provide a pressure profile. Stirring of the overstanding solution was maintained at 900 rpm to prevent the effects of concentration polarisation. After filtration was completed the thickness of the basement membrane films was measured microscopically as described previously [4].

2.3. Treatment of films

GBM was not treated with hexose prior to film formation as it was found that the basement membrane fragments aggregated preventing the formation of uniform, reproducible films. Films were prepared as above and then washed with phosphate buffer (0.1 M sodium phosphate, 50 mM NaCl, 3 mM NaN_3, pH 7.4). Phosphate buffer was used throughout all the glycation reactions at this high concentration in order to maintain a pH of 7.4 throughout the long incubation times. The remaining buffer was then removed and replaced with 50 ml of buffer containing 100 mM hexose and, when appropriate, metal chelators. 15–20 ml were passed through the filter to ensure that all original buffer was washed from the system. Control cells were incubated with buffer only. The filtration cells were then placed in a 37°C incubator for periods of two weeks for incubations with fructose and six weeks with glucose. At the end of the incubation the overstanding solution was removed and the films washed. Treated films were checked micro-

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Abbreviations: TBS, Tris-buffered saline; BSA, bovine serum albumin; GBM, glomerular basement membrane; EDTA, ethylenediaminetetra-acetic acid; DTPA, diethylenetriaminepenta-acetic acid.

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scopically for the absence of microbial infection prior to the filtration of BSA.

Chemicals and proteins were purchased from Sigma Ltd., London. BSA (globulin free) was used for the filtration studies.

3. Results

Changes in film permeability upon treatment with fructose: six films were formed and the buffer flow rates checked to confirm that all the films had J_v values within 5% of the mean. All six were incubated for 14 days at 37°C, three films in phosphate buffer and three in phosphate buffer containing 100 mM fructose. The permeability properties of the films were assayed using BSA (1 mg/ml). Films treated with fructose showed altered permeability from the controls which were incubated in buffer alone. There was a large decrease in the rejection of BSA (Fig. 1A), a sharp increase in protein flux (J_s) and a rise in water flux (J_v) compared with films incubated in buffer alone (Fig. 1B). There was no change in film thicgkness for films that had been treated with fructose, 8.1 ± 1.3 μm compared to 7.9 ± 1.4 μm for control films, showing that the observed changes in permeability were not due to a decrease in film thickness.

3.1. Treatment of films with fructose and metal chelators

The effect of metal ions on the changes in permeability of GBM that occur on incubation with fructose was investigated in two experiments, each with nine GBM films. In the first experiment EDTA (10 mM) was included in the fructose incubation buffer for three of the films, with the remaining films incubated with fructose alone or buffer. The second experiment was identical except that DTPA (10 mM) was included in the place of EDTA. Fig. 2 shows rejection of BSA on films that were treated with buffer, fructose, fructose with EDTA (Fig. 2A) or fructose with DTPA (Fig. 2B). The rejection values for the films treated with fructose and EDTA lay between those of the control films and films treated with fructose alone; EDTA partially inhibited the change. The inclusion of DTPA abolished the change in permeability (Fig. 2).

3.2. Treatment of films with glucose

Incubation with 100 mM glucose for six weeks proved less effective than fructose in terms of promoting changes in the permeability of GBM (Fig. 3). Unlike fructose, glucose treatment did not change the hydraulic permeability of the films but did increase permeability to BSA; in consequence the rejection shown by the glucose treated films fell below that of the control films (Fig. 3). As with fructose no changes in film thickness were observed and the effects of glucose treatment were prevented when DTPA (10 mM) was included in the glucose incubation solution.

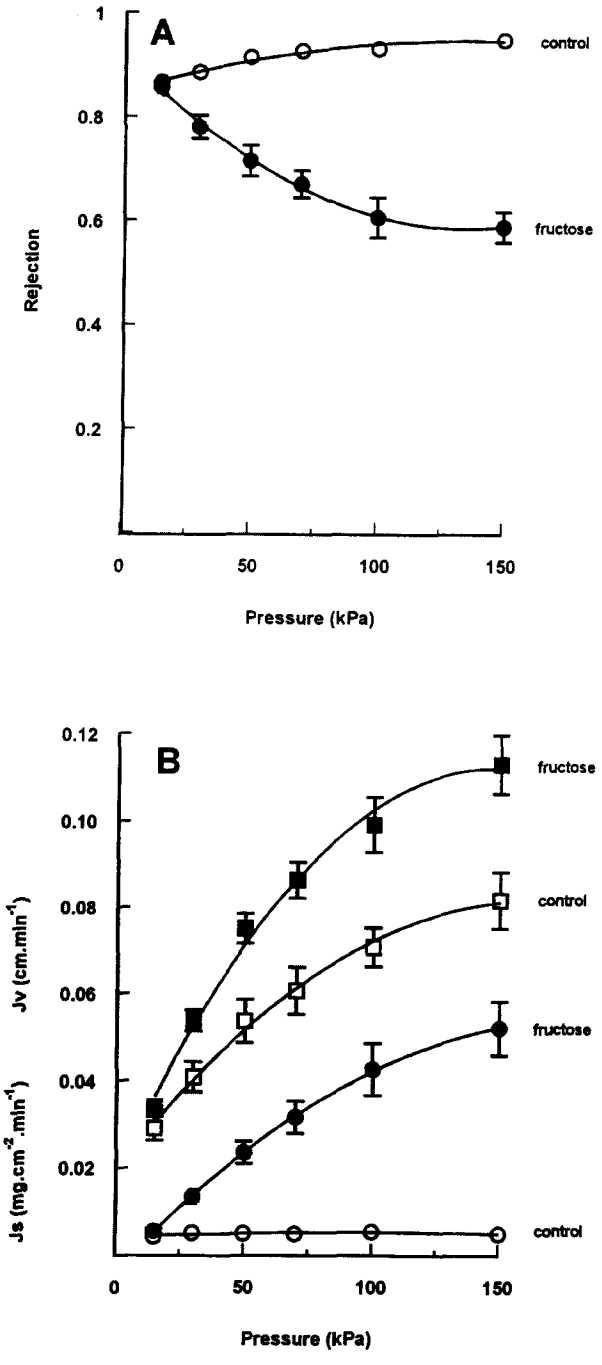

Fig. 1. Ultrafiltration of BSA at different filtration pressures on GBM films treated with fructose (solid symbols) or buffer (open symbols). (A) Shows protein rejections and (B) shows J_v (water flux, squares) and J_s (solute flux, circles). The bars indicate the standard deviation for three measurements from each of three films. Where the standard deviation is very small the bars are omitted for clarity.

4. Discussion

The permselectivity of basement membrane has been explained by assuming the material is a compressible random fibre matrix [3]; increasing filtration pressure promotes higher rejections as compression of the matrix renders it less permeable to protein. This effect can be observed with the control films (Figs. 2,3). On cross-linking with glutaraldehyde the material becomes less compressible so that rejections tend not to increase as filtration pressure increases [4]. On treatment with reducing sugars a similar effect is seen. At low filtration pressures hexose treated membranes behave similarly to the controls but as filtration pressure is increased the hexose treated membranes show increased fluxes presumably because they are more resistant to compression and so retain a more open struc-

ture. Following treatment with fructose rejections fall as pressure is increased which may be explained by concentration polarisation. This occurs as the increased solvent flux carries solute to the filter surface where it accumulates, solute flux increases in consequence since this is determined by the concentration at the filter surface [3]. Dispersal of the accumulated solute can be effected by increased stirring. Doubling the stirring rate was found to increase rejection slightly at high filtration pressures using fructose treated films supporting the view that filtration at high J_v was being influenced by polarisation.

The development of leaks in films would increase both J_v and

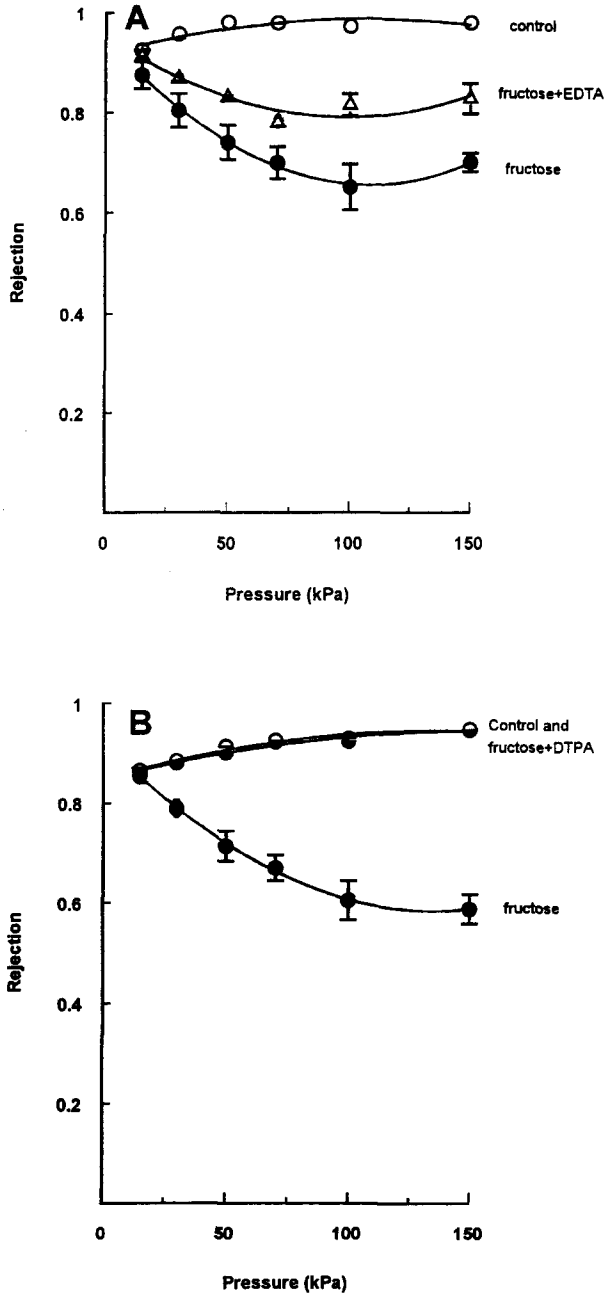

Fig. 2. Rejections of BSA at different filtration pressures on GBM films treated with buffer, fructose and fructose + EDTA (Fig. 2A) or fructose + DTPA (Fig. 2B). The bars indicate the standard deviation for three measurements from each of three films. Where the standard deviation is very small the bars are omitted for clarity.

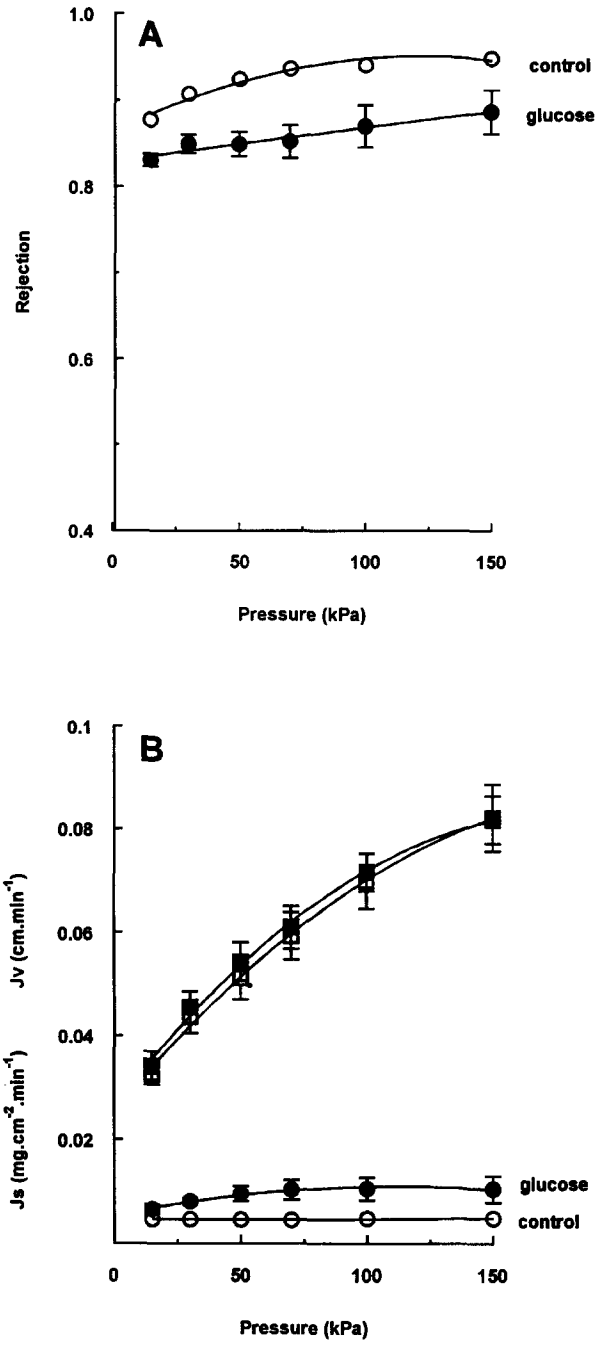

Fig. 3. Ultrafiltration of BSA at different filtration pressures on GBM films treated with glucose (solid symbols) or buffer (open symbols). Fig. 3A shows protein rejections and Fig. 3B shows J_v (water flux, squares) and J_s (solute flux, circles). The bars indicate the standard deviation for three measurements from each of three films. Where the standard deviation is very small the bars are omitted for clarity.

J_s. However, in repeated experiments only fructose treated films showed reproducible and marked changes in permeation properties. Control films, glucose treated films, and films incubated with fructose in the presence of DTPA showed no comparable changes. It seems unlikely that fortuitous leaks should develop only in the fructose treated films, rather it appears that fructose has a specific effect on GBM permeation. The results with glucose support this conclusion; though J_s had increased

J_v remained unchanged indicating no leakage. Film permeability towards macromolecules had increased independently from hydraulic permeability.

An earlier report showed that the GBM underwent no change in permeability following treatment with glucose [2]. In contrast the studies here demonstrate that changes in GBM permeability occurred when films were incubated with fructose and that glucose could elicit a change when at high concentration for prolonged incubation times. A reason for the difference in findings is that Daniels et al. included the metal chelator EDTA in their GBM incubations and we have shown this to be an inhibitor of permeability changes. Additionally Daniels used lower glucose concentrations for briefer times; under these conditions changes are difficult to detect.

It is well established that fructose crosslinks proteins more readily than glucose [5,6]. The identities of the cross-links formed are not well understood [7]. The behaviour of fructose treated basement membrane films is reminiscent of that of glutaraldehyde altered membrane, leading us to suggest that the changes in the BM structure caused by the fructose may be due to the formation of crosslinks. DTPA inhibited the fructose-induced changes in permeability completely, and EDTA partially inhibited these changes, showing that the reactions involved in altering the permeability of the basement membrane were metal catalysed; DTPA is possibly a more effective chelator than EDTA since DTPA suppresses transition metal redox potentials more effectively [8]. This inhibition of the changes in GBM permeability shows that they do not result directly from the formation of Amadori product as this is not affected by the metal chelator DTPA [9]. It is more likely that the changes are brought about by metal catalysed late-glycation reactions such as have been observed in the crosslinking of rat tail collagen where glycoxidative crosslinking was shown to be dependent upon the presence of transition metal ions [9]. Glycoxidative crosslinking could explain the results reported here.

While fructose levels in the kidney in diabetic animals have been reported to be elevated the elevation was not marked, around 80%, and concentration on a wet weight basis was about 1 mM [10]. In the same study glucose concentration was found to be higher, 40 mM. The finding here that incubation with fructose causes more marked changes than glucose suggests that even low levels of fructose, if persistently present, may affect GBM function in vivo.

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References

[1] The diabetes control and complications trial research group (1993) N. Engl. J. Med. 329, 977–986.
[2] Daniels, B.S. and Hauser, E.B. (1992) Diabetes 41, 297–304.
[3] Robinson, G.B. and Walton, H.A. (1989) Microvasc. Res. 38, 36–48.
[4] Walton, H.A., Byrne, J. and Robinson, G.B. (1992) Biochim. Biophys. Acta 1138, 173–183.
[5] Suarez, G., Rajaram, R., Oronsky, A.L. and Gawinowicz, M.A. (1989) J. Biol. Chem. 264, 3674–3679.
[6] Shin, D.B., Hayase, F. and Kato, H. (1988) Agric. Biol. Chem. 52, 1451–1458.
[7] Bailey, A.J., Sims, T.J., Avery, N.C. and Halligan E.P. (1995) Biochem. J. 305, 385–390.
[8] Miller, D.M., Buettner, G.R. and Aust, S.D. (1990) Free Rad. Biol. Med. 8, 95–108.
[9] Fu, M.X., Wells-Knecht, K.J., Blackledge, J.A., Lyons, T.J., Thorpe, S.R. and Baynes, J.W. (1994) Diabetes 43, 676–683.
[10] Tomiya, N., Suzuki, T., Awaya, J., Mizuno, K., Matsubara, A., Nakano, K. and Kurono, M. (1992) Anal. Biochem. 206, 98–104.